

DEVELOPMENTAL CHANGES IN ANTIOXIDANT  
ENZYMES AND THE EFFECTS OF HIGH O<sub>2</sub> AFTER  
BIRTH ON ENZYME ACTIVITY

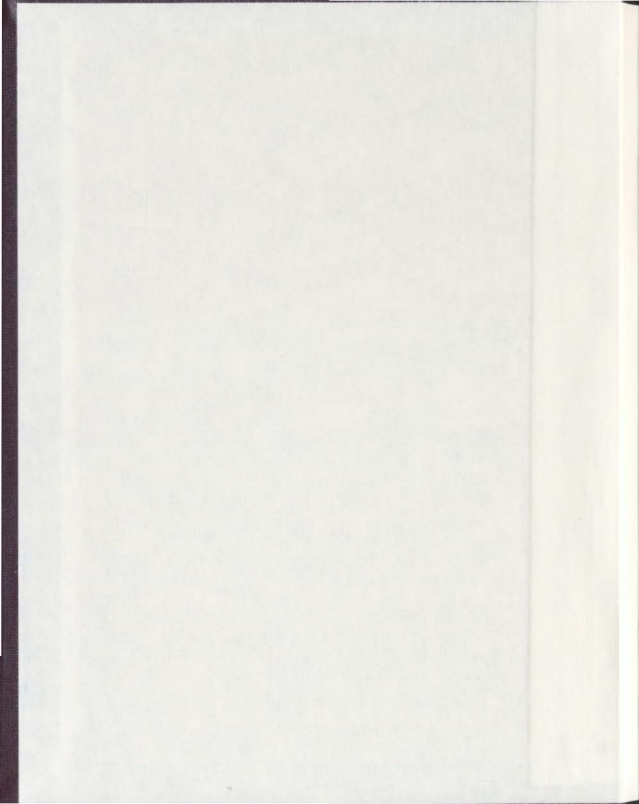
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# **Developmental Changes in Antioxidant Enzymes and the Effects of High O<sub>2</sub> After Birth on Enzyme Activity**

By Zhao Yong

A thesis submitted to the School of Graduate Studies  
in partial fulfillment of the requirements for the degree  
of Master of Science

Department of Biochemistry

Memorial University of Newfoundland

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## ABSTRACT

Antioxidant enzymes play a very important role in protecting newborn babies who have to cope with an increase in  $O_2$  exposure after birth. Some researchers believe that the rise in antioxidant enzyme activity in the last stage of development of fetal life is an essential preparation for birth. However, there are contradictory reports. We have investigated the developmental changes in catalase (CAT), superoxide dismutase (both Mn-SOD and Cu/Zn-SOD) and glutathione peroxidase (GPx) activities in fetal rat livers at different stages of gestation and explored the effects of high  $O_2$  after birth on antioxidant enzyme activities. Our results showed that CAT and GPx activities increased when enzyme activities were expressed per mg protein from 15 day of gestation to 22 day of gestation. But, when SOD activity is expressed per mg protein, total SOD activity decreased. High  $O_2$  exposure induced an increase in AOE activities when newborn rats were exposed to 95%  $O_2$  for 9 days. Both CAT and GPx activities increased continuously. However, the pattern of increasing SOD activity was different from that seen for both CAT and GPx. Total SOD activity rose by 20% at 3 days of exposure, by 34% at 6 days of exposure and by only 9% at 9 days of exposure when compared with corresponding newborn rats exposed to room air. Cu/Zn-SOD activity increased during the first 6 days of exposure and then decreased. There was no change in MnSOD activity during the first 3 days of exposure, but after 3 days of exposure, MnSOD activity began to increase.

In conclusion: 1) both CAT and GPx activities increased during the last stage of development, 2) total SOD activity decreased before birth, 3) total SOD activity did not change concomitantly with both CAT and GPx activity during the last stage of development after birth, 4) all liver antioxidant enzyme activities increased significantly during exposure to high  $O_2$ , but total SOD enzyme activities decreased after having increased for 6 days.

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## List of Abbreviations

- AD – Alzheimer's disease
- ALS – Amyotrophic Lateral Sclerosis
- AMs – Alveolar macrophages
- AOE – antioxidant enzyme activity
- AT – 3-amino-1,2,4-triazole
- CAT – catalase
- Cu/Zn-SOD – a copper- and zinc-containing superoxide dismutase
- DDC – Diethyldithiocarbamate
- DS – Down Syndrome
- DSPC – disaturated phosphatidylcholine
- GPx – glutathione peroxidase
- GSH – Reduced glutathione
- GSSG – Glutathione disulfide
- H<sub>2</sub>O<sub>2</sub> – hydrogen peroxide
- LPO – lipid peroxidation
- Mn-SOD – a manganese-containing superoxide dismutase
- OH – hydroxyl radical
- O<sub>2</sub> – superoxide anion
- ROS – Reactive oxygen species
- SD – Standard deviation
- TNF – Tumour necrosis factor
- TGF – Transforming growth factor

## Chapter 1: Literature Review

### 1.1 Antioxidant enzymes

Small amounts of reactive oxygen species (ROS), such as hydroxyl radicals ( $\cdot\text{OH}$ ), superoxide anions ( $\text{O}_2^{\cdot-}$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), are constantly generated in aerobic organisms in response to both external and internal stimuli (1). Low levels of ROS may be indispensable in many processes (2), including intracellular signal transduction for cell proliferation or apoptosis, immunity, or defense against microorganisms. In contrast, high doses and/or inadequate removal of ROS results in oxidative stress, which may cause severe metabolic malfunctions and damage to biological macromolecules. A wide array of enzymatic antioxidant defenses exists in the body, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). These antioxidant enzymes protect cells from oxygen toxicity.

The steady-state levels of ROS are determined by the rate of ROS production and their clearance by scavenging mechanisms (3). Certain antioxidant enzymes, including superoxide dismutase, glutathione peroxidase, catalase, and thioredoxin are potent ROS scavengers but occur in cells only at relatively low concentrations. The same is true for nonenzymic antioxidants such as amino acids and proteins. Amino acids are less effective than the classical antioxidants on a molar basis, but their cumulative intracellular concentration is very high.

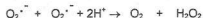
#### 1.1.1 Superoxide dismutase

Superoxide dismutase destroys the free radical superoxide by converting it to peroxide

that can in turn be destroyed by CAT or GPx reactions. SOD converts the highly reactive superoxide radical to the less reactive  $H_2O_2$ .

There are three genetically distinct mammalian isoforms of SOD (4, 5). They are cytosolic CuZn-SOD or SOD-1, mitochondrial Mn-SOD or SOD-2, and extracellular EC-SOD or SOD-3. SOD-1, a copper- and zinc-containing SOD, is localized primarily in cytoplasmic and nuclear compartments (6). SOD-2, a manganese-containing SOD is found predominantly in mitochondria (7). SOD-3 is the predominant extracellular antioxidant enzyme and has been found in serum and in cerebrospinal, ascitic, and synovial fluids (8). In some human tissues such as the uterus, umbilical cord, placenta, and arteries, EC-SOD enzyme activity equals or exceeds that of CuZn-SOD and Mn-SOD (9,10).

Superoxide dismutase is the antioxidant enzyme that catalyses the dismutation of the highly reactive superoxide anion to  $O_2$  and to the less reactive species  $H_2O_2$ . Peroxide can be destroyed by both CAT and GPx (11).



SOD destroys  $O_2^{\cdot -}$  by successive oxidation and reduction of the transition metal ion at the active site in a ping pong type mechanism with remarkably high reaction rates. All types of SOD bind single charged anions such as azide and fluoride, but distinct differences have been noted in the susceptibilities of Fe-, Mn- or CuZn-SODs. CuZn-SOD is competitively inhibited by,  $CN^-$ (12), and by  $F^-$ (13).

#### 1.1.1.1 Mn-SOD

Mn-SOD is a homotetramer (96 kDa) containing one manganese atom per subunit that cycles from Mn (III) to Mn (II) and back to Mn (III) during the two step dismutation of superoxide (14). The respiratory chain in mitochondria is a major source of oxygen

radicals, so, Mn-SOD plays a very important role in scavenging  $O_2$  at that location. Mn-SOD has been shown to be greatly induced by cytokines, but is only moderately influenced by oxidants (15). The biological importance of Mn-SOD also includes; (a) preventing mutation: Farr et al (16) reported that inactivation of Mn-SOD genes in *Escherichia coli* increased mutation frequency when grown under aerobic conditions, (b) decreasing the sensitivity to oxidative stress: Van loon et al (17) reported that elimination of the gene in *Saccharomyces Cerevisiae* increased its sensitivity to oxygen and transfection of Mn-SOD cDNA into cultured cells rendered the cells resistant to paraquat, TNF and Adriamycin-induced cytotoxicity, and radiation induced-neoplastic transformation (18). Wispe et al utilized expression of human Mn-SOD genes in transgenic mice and proved that Mn-SOD can protect against oxygen-induced pulmonary injury and Adriamycin-induced cardiac toxicity (19), (c) protecting heart and neonatal development: Li et al (20) reported that lack of expression in Mn-SOD knock-out mice results in dilated cardiomyopathy and neonatal lethality.

#### 1.1.1.2 CuZn-SOD

CuZn-SOD (21, 22) is believed to play a major role as the first line of antioxidant defense. Calves that were fed milk supplemented with 25 ppm Cu and 100 ppm Zn showed a stronger immune response and a higher SOD activity than calves that were not (23). Other recent reports (20) involving SOD knock-outs have revealed that Mn-SOD is essential for life whereas CuZn-SOD is not. CuZn-SOD knock-out mice appear normal and exhibit differences only after traumatic injury, whereas Mn-SOD knockouts do not survive past 3 weeks of age (20). In a variety of human tissues, Mn-SOD content was reported to be roughly one-half that of the CuZn-SOD content (24).

#### **1.1.1.3 EC-SOD**

Extracellular superoxide dismutase is a secretory tetrameric, copper and zinc containing glycoprotein with a high affinity for certain glycosaminoglycans. EC-SOD was found in the interstitial spaces of tissues and also in extracellular fluids, accounting for the majority of the SOD activity in plasma, lymph, and synovial fluid (4, 24). It is well documented that under conditions of hyperoxia, the lungs are exposed to oxidative stress, leading to both endothelial and epithelial injury. Recently, mice totally lacking EC-SOD have been created and they showed evidence of increased sensitivity to hyperoxic exposure. When hEC-SOD is overexpressed in a highly specific fashion in airway and alveolar epithelial cells, these animals have an increased tolerance to hyperoxia. EC-SOD is not induced by its substrate nor by other oxidants and its regulation in mammalian tissues primarily occurs in a manner coordinated by cytokines, rather than as a response of individual cells to oxidants (25,26).

#### **1.1.1.4 Diseases related to SOD**

##### **1.1.1.4.1 SOD-1 and Down syndrome**

It is widely thought that the specific deficits observed in Down syndrome (DS) patients are caused by the overexpression of specific genes on chromosome 21. This region contains genes encoding the cytoplasmic copper and zinc containing superoxide dismutase. There are reasons to believe that genes encoding SOD-1 might play some role in the etiology of DS (25).

Mice that overexpress SOD-1 to varying degrees have been reported to exhibit some DS-like characteristics (27). SOD-1 has an extremely important role in the cellular defense

system against damage by oxygen radicals. Since its primary action is the formation of hydrogen peroxide from the superoxide anion, an overexpression of SOD-1 could lead to an increase in basal hydrogen peroxide concentration. Since hydrogen peroxide can decompose to form the very reactive hydroxyl radical (28), it is possible that overexpression of SOD-1 could increase basal oxidative stress.

It has also been shown that normal SOD-1 participates in side reactions which may result directly in the peroxidation of cellular components and the nitration of tyrosine residues. Weidau-Pazos et al found that wild type SOD-1 can react with the paramagnetic resonance spin trap 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO) (29). Although DMPO is not normally found in mammalian tissue, its size and structure indicates that biological molecules could undergo the same reactions. Increasing SOD-1 levels could thus lead to a direct increase in peroxidation of certain cellular constituents. SOD can also directly react with peroxynitrite through its copper-containing reaction center forming a nitronium-like compound which can in turn cause the nitration of nearby tyrosine residues (30, 31). Overexpression of SOD-1 may also upset cellular copper homeostasis, leading to an increased free copper load and a consequent increase in copper-mediated nitration of tyrosines (32). It has been shown that increased free copper is responsible for other neurologic disorders such as Wilson's disease and Menkes' syndrome (33, 34). These examples of SOD-1 overexpression leading to increases in lipid peroxidation, DNA strand breakage, protein carbonylation and nitration are potentially fatal to the cell and, in a larger sense, could cause some of the symptoms associated with DS.

In addition, the superoxide anion is used as an intermediate in certain cellular reactions (35). It is possible that a basal steady state intracellular concentration of superoxide anion is required for the maintenance of cellular viability. Much research shows that superoxide generating systems are toxic to most cell types, and that superoxide scavenging systems

are protective, but there is little to indicate that intracellular superoxide concentration can be eliminated completely without some unforeseen consequences. Many enzymes use superoxide as an intermediate and these reactions are tightly coupled. Some steady state superoxide concentration may be required for optimal cellular functioning. The action of the gaseous second messenger/ neurotransmitter nitric oxide is terminated by superoxide (36). If this is a physiological process, then removal of superoxide from the intra- and intercellular milieu could adversely affect central nervous system function and perhaps have vascular effects as well.

#### **1.1.1.4.2 SOD-1 and Amyotrophic Lateral Sclerosis**

Certain mutations in SOD-1 are also responsible for a familial form of the neurodegenerative disease, Amyotrophic Lateral Sclerosis (ALS,) (37). These mutations are invariably dominant, implicating a gain of function by SOD-1 in the etiology of the inherited disorder. The mutations may result in a decrease in activity of the enzyme, but they are all hypothesized to decrease the specificity of the enzyme and increase the range of substrates upon which it can act (38). In particular, it is thought that nitric oxide, a nitroso-radical neurotransmitter/ second messenger, can be acted upon to form a highly reactive and toxic radical, possibly peroxynitrite. This molecule can then either directly attack tyrosine residues on nearby proteins or break down to form another highly toxic radical species (presumably the hydroxyl radical) and interfere with intracellular homeostasis (39). Importantly, it has been reported that this reaction can occur even in the presence of normal SOD-1 (40). Similarly, the reaction of SOD-1 with DMPO to form DMPO-OH is greatly accelerated by the same mutations that cause ALS. These mutations may thus lead to greatly increased side reaction activity of SOD-1 with concomitant production of nitrated



tyrosyl residues in proteins and peroxides in lipids, both of which are highly disruptive to cellular homeostasis and would likely cause cell death (41). In addition, increased oxidative stress may play a role in inactivating glial glutamate transport. Thus the mutations causing ALS may initiate a feed-forward cycle of oxidative stress leading to increased glutamate toxicity, then further oxidative stress, and finally death.

The relationship of SOD-1 to sporadic ALS is not very clear. It is most likely that SOD-1 and the other antioxidant systems are somehow unable to adequately protect the motor neurons from whatever insult is present - possibly an increase in non-NMDA glutamate receptor activation. The rescue from degeneration in bcl-2 overexpressing transgenic mice (42) argues for this interpretation. Alternatively, excess stimulation of cells might lead to abnormal substrate presentation to SOD-1 and cause changes in cellular homeostasis and consequent cell death.

## **1.1.2 Catalase**

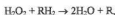
Catalase is one of the most potent catalysts known. The reactions it catalyses are crucial to life. Catalase catalyses the conversion of hydrogen peroxide, a powerful and potentially harmful oxidizing agent, to water and molecular oxygen (43). Catalase also uses hydrogen peroxide to oxidize toxins including phenols, formic acid, formaldehyde and alcohols. In addition, catalase is a heme-containing redox enzyme that is found in high concentrations in the peroxisome (44).

### **1.1.2.1 Major functions of catalase**

Catalase catalyzes the following reaction;  $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ ,

$\text{H}_2\text{O}_2$  is a powerful oxidizing agent and is potentially damaging to cells. By preventing excessive  $\text{H}_2\text{O}_2$  build up catalase allows important cellular processes which produce  $\text{H}_2\text{O}_2$  as a byproduct to take place safely (45).

Catalase performs a very elegant 'reshuffling' of toxic compounds. This enzyme can also act as a peroxidase for which several organic substances, especially ethanol, can act as a hydrogen donor. Enzymes from some microorganisms, such as *Penicillium simplicissimum*, which exhibit both catalase and peroxidase activity, have sometimes been referred to as catalase-peroxidase. In the following peroxidative reaction, a second family of reactions catalyzed by catalase, include phenols, formic acid, formaldehyde and alcohols (46):



Hydrogen peroxide breakdown and the peroxidative reaction consume  $\text{H}_2\text{O}_2$ . Catalase activity in the cell is therefore important (47) for peroxidation reaction. Peroxisomes partially oxidize fatty acids, producing  $\text{H}_2\text{O}_2$  as a byproduct. This peroxisomal oxidation shortens the fatty acids to length C8 or longer and facilitates an energy efficient degradation in the mitochondrion. The peroxisomal oxidation is slightly less efficient in ATP production than the mitochondrial oxidation. However, it is less of a waste of available energy than it might seem. Some of the 'missing energy' is locked up in the oxidative power of  $\text{H}_2\text{O}_2$  which is used in the peroxidative reaction (48). Another redox reaction which indirectly involves catalase concerns the production of DNA (49). Ribonucleotide reductase is responsible for conversion of ribonucleotide diphosphates to their corresponding deoxyribonucleotide diphosphates. Ribonucleotide reductase has a tyrosyl free radical which is essential to its action. This radical is produced by an enzyme, NAD(P)H:flavin oxidoreductase, which releases a superoxide ion,  $\text{O}_2^{\cdot-}$  and this highly

reactive radical is converted to  $\text{H}_2\text{O}_2$  by the action of superoxide dismutase:



The  $\text{H}_2\text{O}_2$  generated is then a substrate for catalase. Superoxide and hydrogen peroxide can lead to further production of oxygen radicals, which can cause lipid peroxidation and disrupt metabolic processes.

Oxygen radical stress occurs in all aerobic organisms as a result of aerobic metabolism and increased free-radical formation (oxidative stress) is likely to increase tissue damage (50). Catalase has been shown to be very effective in hydrogen peroxide detoxification. Catalase is also involved in signal transduction (51) with more and more research showing that  $\text{H}_2\text{O}_2$  acts as secondary messenger in cell signal transduction and gene expressions. Stimulation of human lung fibroblast cells with TGF-beta-1 resulted in a transient burst of reactive oxygen species with a maximal increase 5 min after treatment. This increase in reactive oxygen species was inhibited by catalase. TGF-beta-1 treatment stimulated IL-6 gene expression and protein synthesis in human lung fibroblast cells. Antioxidants including NAC, glutathione, and catalase reduced TGF-beta-1-induced IL-6 gene expression, and direct  $\text{H}_2\text{O}_2$  treatment induced IL-6 expression in a dose-dependent manner. Catalase also reduced TGF-beta-1-induced AP-1 binding activity, which is involved in IL-6 gene expression. It has been reported that  $\text{Ca}^{2+}$  influx is stimulated by TGF-beta-1 treatment. Catalase suppressed TGF-beta-1 or  $\text{H}_2\text{O}_2$ -induced IL-6 expression, and ionomycin increased IL-6 expression, simultaneously modulating AP-1 activity.  $\text{H}_2\text{O}_2$  treatment induced an increase in  $[\text{Ca}^{2+}]$  for AP-1 activity and signaling in rat fibroblasts. Ionomycin, the calcium ionophore, also induced an increase in  $[\text{Ca}^{2+}]$  through activation of calcineurin. Therefore, the authors believed that  $\text{H}_2\text{O}_2$  and ionomycin demonstrate the same pattern in activating AP-1 and signalling. PD 98059, an inhibitor of mitogen-activated

protein kinase (MAPK) kinase/extracellular signal-related kinase-1, suppressed TGF-beta-1 or H<sub>2</sub>O<sub>2</sub>-induced IL-6 and AP-1 activation. In addition, TGF-beta-1 or H<sub>2</sub>O<sub>2</sub> increased MAPK activity which was reduced by catalase, suggesting that MAPK is involved in TGF-beta-1 induced IL-6 expression. Taken together, these results suggest that TGF-beta-1 induces a transient increase of intracellular H<sub>2</sub>O<sub>2</sub> production, which regulates downstream events such as Ca<sup>2+</sup> influx, MAPK, AP-1 activation and IL-6 gene expression. As catalase can degrade H<sub>2</sub>O<sub>2</sub> and control the level of H<sub>2</sub>O<sub>2</sub>, catalase may play an important role in signal transduction.

Catalases fall into two main classes (52), the HPI and HPII Catalases. HPII Catalases catalyze just the breakdown of H<sub>2</sub>O<sub>2</sub> and HPI Catalases catalyze both reactions. HPI Catalases exist as two isozymes, HPI-A and HPI-B.

#### **1.1.2.2 Diseases related to catalase**

Evidence is accumulating that many human degenerative diseases may have their origin in deleterious free radical reactions (53). These diseases include atherosclerosis, cancer, inflammatory joint disease, asthma, diabetes, senile dementia, Parkinson's disease, and degenerative eye disease.

##### **1.1.2.2.1 Ageing**

The process of biological ageing might also have a free radical basis. Most free radical damage to cells involves oxygen free radicals or, more generally, reactive oxygen species (ROS) which include non-radical species such as singlet oxygen and hydrogen peroxide as well as free radicals. ROS can damage genetic material, cause lipid peroxidation in cell membranes, and inactivate membrane-bound enzymes. Humans are well endowed with antioxidant defenses against ROS; these antioxidants, or free radical scavengers, include

ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), beta-carotene, coenzyme Q10, enzymes such as catalase and superoxide dismutase, and trace elements including selenium and zinc (54). The eye is an organ with intense ROS activity, and it requires high levels of antioxidants to protect its unsaturated fatty acids.

#### **1.1.2.2.2 Hereditary catalase deficiencies and diabetes.**

Partial or near-total lack of erythrocyte catalase activity is a rare condition, generally thought to be benign (55). However, little is known of the frequency of common diseases of adult onset in human beings with catalase deficiency (56a). In a series of Hungarian patients with catalase deficiency, there is a higher frequency of diabetes than in unaffected first-degree relatives and the general Hungarian population (55). It is speculated that quantitative deficiency of catalase might predispose to cumulative oxidant damage of pancreatic beta-cells and diabetes. Hydrogen peroxide is a ubiquitous oxidant, generated as a byproduct of normal cellular respiration and from sources such as activated phagocytes and peroxidogenic bacteria. Almost all aerobic organisms have enzymes such as catalase designed to clear hydrogen peroxide and related organic hydroperoxides, so it is surprising that occasional instances of hypocatalasaemia or even acatalasaemia are reported in human beings (56a). The first cases presented with gangrenous gum disease, which often led to complete edentulation (57a). Similar patients (with different molecular causations of catalase deficiencies) have been reported from Switzerland and Hungary, but were free of any pathological complications of the deficiency (55). It has, therefore, become almost dogma that partial or near-total catalase deficiency is not associated with any important pathological complication. However, common adult-onset diseases such as diabetes and

atherosclerosis have not been studied in relation to catalase deficiency.

#### **1.1.2.2.3 Down syndrome**

Down syndrome (trisomy 21) is the most common genetic cause of mental retardation (58a). There are several lines of evidence showing that individuals with Down syndrome (DS) are under unusual oxidative stress (59a). First, they age prematurely and develop Alzheimer-like changes in their brains in their 30s or 40s and many become demented (60a). Furthermore, they suffer from premature onset of degenerative pathologies with a free radical component, such as cataracts and autoimmune diseases. Second, DS neuron degeneration in vitro is completely averted by treatment with free radical scavengers, such as the antioxidant vitamins (vitamin E and C), or catalase (60a). Oxidative stress may result from excess of the enzyme CuZn superoxide dismutase activity (increased in individuals with Down syndrome by ~50% (61a). Indeed, the ratio of SOD-1 to CAT plus GPx is altered, meaning that more potentially damaging hydrogen peroxide is generated by SOD-1 than CAT and GPx can neutralize, leading to severe oxidative imbalance (61a).

#### **1.1.2.3 Future Prospects**

Researchers at Columbia University have discovered an enzyme that is required to prolong the life-span of microscopic roundworms; strains of long-lived worms appear to produce this enzyme in greater quantity than normal (56). They believed the enzyme, cytosolic catalase, protects cells from oxidative damage and considered it as a key element in the aging process in all animals, including humans. Because oxidative damage has been implicated in Alzheimer's and ALS, these results may prompt medical researchers to ask new questions about such nervous system diseases.

### 1.1.3 Glutathione peroxidase

The selenium-containing glutathione peroxidase contains a single selenocysteine residue in each of the four identical subunits, which is essential for enzyme activity (57). GPx catalyses the reduction of hydroperoxides using glutathione (GSH), thereby protecting mammalian cells against oxidative damage. In fact, glutathione metabolism is one of the most important antioxidant defense mechanisms (58, 59).



#### 1.1.3.1 Classification, distribution and functions

There are five GPx isoenzymes found in mammals. Although their expression is ubiquitous, the levels of each isoform vary depending on the tissue type (60). Cytosolic and mitochondrial glutathione peroxidase (c-GPx or GPx1) are widely distributed and their primary function is to counteract oxidative attack. The gastrointestinal isoenzyme (GI-GPx or GPx2) is most related to c-GPx and is exclusively expressed in the gastrointestinal track (72). It might provide a barrier against hydroperoxides derived from the diet or from metabolism of ingested xenobiotics. The plasma GPx (p-GPx or GPx3) behaves similarly to GPx in selenium deficiency. It is directed to extracellular compartments and is expressed in various body fluids. Phospholipid hydroperoxide glutathione peroxidase (PH-GPx or GPx4), originally presumed to be a universal antioxidant enzyme protecting lipid membrane, appears to have adopted a variety of specific roles such as silencing lipoxygenases and becoming an enzymatically inactive structural component of the mitochondrial capsule during sperm maturation (73). Recently, a new member, GPx5, was detected specifically in mouse epididymis and is not selenium dependent (61). Although GPx shares the substrate,  $\text{H}_2\text{O}_2$ , with CAT, it alone can react effectively with lipid and other organic hydroperoxides and is the major protection against low levels of oxidant stress.

### c GPx.

Under adequate selenium supply all cells express at least some c-GPx. Particularly high levels are generally found in tissues with a high rate of peroxide production, such as erythrocytes, liver, kidney, and lung (62). In rats, the lowest c-GPx levels were measured in testes. Developmental changes in c-GPx expression have been observed in rat lungs where it increased after birth, especially when exposed to high oxygen tensions (63). In addition, developmental changes in c-GPx expression have also been observed in rat intestine. Two weeks after birth, c-GPx protein became undetectable in the duodenum and disappeared from the ileum after weaning. Thus, with some exceptions, c-GPx expression parallels metabolic activity, which is consistent with an antioxidant function of the enzyme. Nevertheless, the increasing knowledge of the role of hydroperoxides in cellular signaling has drawn attention to glutathione peroxidase as a putative regulator.

Potential roles of c-GPx in modulating NF- $\kappa$ B activation include: a) inhibiting activation of NF- $\kappa$ B by inhibiting cyclo-oxygenases and lipoxygenases (64); b) regulating the activity of cyclo-oxygenases (65); c) or by controlling the levels of the hydroperoxide involved in counteracting hydroperoxide-modulated apoptosis (66, 67). Apoptosis can be induced in T cells by Fas-antigen or TNF, but also by reactive oxygen species (68,69). Because these are sufficient to trigger apoptosis, they have been suggested to constitute intracellular signals common to apoptotic processes in general. Accordingly, c-GPx activity, enhanced either by supplementation of bovine renal epithelial cells with selenium or by overexpression of c-GPx in a myeloid cell line, inhibited apoptosis induced by hydroperoxides.

One way of determining the physiologic function of an enzyme is to "knock it out" and study the consequences. Two strains of c-GPx knockout mice have been created (70). These mice grew and developed normally and did not show any histopathologies up to 15



months of age indicating a limited role of c-GPx during normal development and under physiologic condition. Furthermore, these mice were not affected by exposure to hyperbaric oxygen. However, when stressed with paraquat, c-GPx(-/-) mice died faster than controls (71). Franco et al (71) found that treatment with the pro-oxidant paraquat resulted in time and dose-dependent increases of transcription levels for GPx. If GPx is knocked out, mice die faster than controls suggesting that GPx plays a role in fighting oxidative stress.

#### **GI-GPx,**

The expression of the gastrointestinal GI-GPx appears to be restricted to the epithelium of the gastrointestinal tract in rats (72), whereas in humans it is also found in liver. GI-GPx appears to be the major antioxidant defense of the intestinal epithelium (73). More recently, Chu et al, (74) suggested a possible protective role of GI-GPx against susceptibility to colon cancer by chromosomal mapping of the mouse GI-GPx gene near the Ccs 1 locus on chromosome 12. GI-GPx would balance the mutagenic potential of hydroperoxides and may therefore be viewed as a device to protect against the initiation process in the development of malignancies of the gastrointestinal tract.

#### **p-GPx**

The variant, p-GPx, was first detected in blood plasma and found to be different from c-GPx. The main source of plasma p-GPx is the kidney (75). It is produced by the cells of the proximal tubular epithelium and in the parietal cells of Bowman's capsule and released into the blood. The kidney is the site which contains the highest levels of p-GPx mRNA (76); less is found in liver, skeletal muscle, pancreas, brain, lung, heart, the ciliary epithelium of the eye, the mature absorptive epithelial cells of the large intestine, epididymis, placenta, and mammary gland of pregnant mice. From the site of production, p-GPx is secreted into

the surrounding extracellular environment and has been found in milk (77), amniotic fluid, in lung lavage, in the extracellular milieu of the intestinal epithelium released from the basolateral side of the epithelial cells, and in the aqueous humor.

During human fetal development, p-GPx is synthesized in trophoblast cells of the placenta (78). Developmental expression was investigated by determining the site of p-GPx expression in different embryonic states and in adult tissues of mice (79). In pregnant mice it is found in the decidua, visceral yolk sac, and fetal skin, i.e., at the embryo/maternal interfaces. In adult tissues apart from known sites, like proximal renal tubulus, it was found also in the skin, intestinal villi, and adipose tissue, i.e., tissues that are engaged in active transport or metabolism of lipids.

p-GPx can regulate the levels of extracellular hydroperoxides which are presumed to be relevant to lipid mediator biosynthesis. p-GPx might, thereby, become a key regulator of the host defense reaction. It is reasonable to assume that the normally silenced lipoxygenases initiating prostaglandin and leukotriene synthesis are activated by the oxidative burst of irritated phagocytes. The activation of lipoxygenase would amplify the initial phagocytic response, e.g., by providing chemotaxins and proinflammatory mediator, and end up in a full-blown inflammation if not counteracted. An efficient regulation of this cascade of events could be achieved by a redox buffer that, by silencing lipoxygenases, prevents the amplification of any irrelevant stimulation of a single phagocyte but is overcome if a critical threshold of noxious events is reached that requests an adequate host defense reaction. In fact, a fast extracellular peroxidase like p-GPx with a limited supply of reducing equivalents would be ideal to meet the demands of the systems.

#### PH-GPx

PH-GPx is found in most tissues, especially in the testis (80). The preferential

channeling of selenium into PH-GPx in the reproductive system and the endocrine organ may indicate that PH-GPx not only has antioxidant functions but may also play a role in redox regulation, sexual maturation, and differentiation. PH-GPx was discovered as factor preventing lipid peroxidation (81) and primarily discussed as the glutathione peroxidase that protects biomembranes against oxidative stress. Its ability to reduce hydroperoxides in HDL and LDL (82) provides the basis for ongoing discussions on its relevance to atherogenesis. PH-GPx can accept not only hydroperoxides from complex lipids and even membranes, but may also react with thiols in bulky molecules, like those present in proteins, leading to an alternate enzymatic activity of PH GPx: a "thiol oxidase activity" (83) towards thiols in proteins.

#### **GPx5**

GPx5 is a secreted sperm-bound selenium-independent glutathione peroxidase protein which is found only in specific regions of the epididymidis. Because of the lack of selenium (Se) in the active site of this enzyme, unlike the other animal GPx characterized to date, it was suspected that GPx5 does not function in the epididymis as a true glutathione peroxidase *in vivo* (83a). But, recent research shows that the selenium-independent GPx5 could function as a back-up system for Se-dependent GPxs (83a).

#### **1.1.3.2 Diseases related to GPx**

Cells depleted of glutathione peroxidase were more sensitive to the toxicity of paraquat and adriamycin than untransfected parental cells from which they derived (83). These data strongly suggest that the enzymatic action of GPx protects cells from the toxicity of paraquat and adriamycin. The toxin that these agents induce is likely to be hydrogen peroxide or another hydroperoxide upon which glutathione peroxidase acts.

There are many diseases related to reactive oxygen species. These ROS are generated during metabolism and can enter into reactions that, when uncontrolled, can affect certain processes leading to clinical manifestations (84). Direct effects include peroxidative changes in membranes and other cellular components, including oxidative DNA damage. GPx works with SOD and CAT within cells and remove these ROS before they result in damage to cells. An imbalanced production of ROS plays a role in the pathogenesis of a number of human diseases such as ischemia/reperfusion injury, atherosclerosis, neurodegenerative diseases, cancer, and allergy (85,86). In addition, an imbalance in antioxidant enzymes has been suggested to occur with many other specific pathologies as chronic granulomatous disease, Down Syndrome, diabetic complications, hepatitis, rheumatoid arthritis, Influenza, ulcer, pneumonia, cataract and glaucoma (85).

## **1.2 The Developmental changes of antioxidant enzymes**

Development entails an increased metabolic rate and regional variations in the rate of oxygen utilization by tissues. This metabolic gradient promotes the generation of active oxygen species (86, 87), such as hydrogen peroxide; hydroxyl radical and superoxide anion. There are many known cellular reactions in which active oxygen species damage cellular constituents (88). Free-radical generation by various metabolic pathways could govern some developmental changes (89). During the fetal-neonatal transition, dramatic changes in the  $O_2$  partial pressure in lung and blood cells occur, due both to the cardiovascular changes and to the high  $O_2$  pressure in the atmospheric air. These changes in  $O_2$  availability may result in oxidative stress to cells (90). But, at the same time, the upsurge in the rate of generation of cellular oxidants (91) stimulates an increase in the activities of antioxidant enzymes. The antioxidant enzymes can serve to protect the organism from ROS.

Frank et al (92) measured the activities of antioxidant enzymes in the developing lungs

of rabbit fetuses from 10 days before parturition to several days after birth. They found that SOD, CAT, and GPx activities showed significant increases in activity during the last 3-5 days before birth compared with earlier gestational levels. During the final days *in utero*, SOD and CAT activities increased by approximately 110 % and lung GPx activity by approximately 200 %. There were no parallel changes in lung O<sub>2</sub> consumption demonstrable over this same prenatal period. They suggested that the few days before birth were the key stage for the developing lung of rabbit fetuses to produce enough antioxidant enzymes to prepare for the high oxygen environment they would face after birth.

Frank et al (93) also examined the chronology of development of fetal lung antioxidant enzymes (SOD, CAT, and GPx) during late gestation in three other laboratory animal species: rat, hamster, and guinea pig. They found an essentially similar pattern of prenatal biochemical maturation. The developmental changes were characterized by rapid elevations in fetal lung antioxidant enzyme levels during the final 10% to 15% of gestation. The increase in the lung activity of the individual antioxidant enzymes prior to birth averaged approximately 150% to 200%. The increase led to the suggestion that the induction of pulmonary antioxidant enzymes is precisely regulated and can be considered to be part of a general preparation for birth. Hayashibe et al (94) determined antioxidant enzyme capacity from day 18 to 22 of gestation in rat lung, kidney, and heart. They found that the prenatal maturation of antioxidant enzyme capacity occurs earlier in the heart and later in the kidney than in the lung.

Asikainen et al (95) investigated the developmental profile of antioxidant enzymes in human lung and liver. They found that pulmonary CAT activity increased toward term and adulthood and the hepatic activity of MnSOD and CuZnSOD increased toward adulthood. Qanungo et al (96) investigated the ontogenic profile of antioxidant enzymes in human

placenta and fetal liver. Their results showed that CAT, SOD and glutathione reductase activities increased significantly, but GPx activity remained relatively constant throughout development. The activities of the antioxidant enzymes, SOD and CAT in human placental homogenate and fetal liver appear to increase significantly as gestation progresses. In order to meet the increasing demands of the growing fetus, more and more oxygen enters the fetoplacental circulation during ontogeny thus exposing it to a greater threat of oxygen free radicals. An increase in the activity of SOD with the advancement of pregnancy suggests an increased protection of the placenta and fetus against the damaging actions of  $O_2^{\cdot-}$  anions. A steady rise in the activity of CAT through the gestational period is suggestive of a constant protection of these proliferating placental and fetal organs against the deleterious reactions of  $H_2O_2$ . Another observation made in relation to the activity of the two enzymes in placental-fetal tissues is that at any particular stage of intra-uterine development, the highest activities of SOD and CAT have been observed in fetal liver. This is possibly a reflection of the well-known fact that liver is the site for several detoxification reactions in the body that appears to initiate in early fetal life.

However, contradictory results regarding the development of fetal lung AOE have been described by Stranges et al. (97, 98). This group investigated the expression of CuZnSOD and Mn-SOD in lungs of premature infants with hyaline membrane disease and bronchopulmonary dysplasia as well as the development of GPx activities in human lung cytosol. They did not find any difference in activity for CuZn-SOD, MnSOD and GPx in lungs from control and affected infants nor from aborted fetuses (10-24 weeks gestation) or infants who died postnatally after term delivery. Expression of CuZn-SOD and Mn-SOD was similar in these subjects and in the patients who died postnatally. Activity measurements and immunoblotting studies showed continuous expression of these

enzymes throughout development with no apparent change in protein levels or size. They suggested that, unlike some experimental animals, expression of at least these antioxidant enzymes in human infants born prematurely was similar to that in adults, and indicated that such infants were better adapted for life in an oxygen-containing environment than previously suspected. The authors also concluded that the antioxidant enzymes, CuZn-SOD, Mn-SOD and GPx were expressed constitutively throughout gestation and early neonatal life. These observations differ from those reported in an earlier study by Autor et al (99) who found a 3-fold increase in pulmonary SOD activity between 20 wks gestation and term.

McElroy et al (100) also investigated CAT, SOD, and GPx activities in lung and liver during human development. They determined the developmental expression of CAT, Mn-SOD, CuZn-SOD and GPx activities in human lung and liver ranging in age from 10 weeks gestation to 3 months following birth. They did not detect any noticeable change for pulmonary SOD and GPx activities over this period. CAT activity however, increased from 11 to 20 weeks gestation. Lung CAT activity was temporally associated with the late gestational increase in the fractional content of lung dipalmitoylphosphatidylcholine (DPPC). In contrast with the lung, liver total SOD activity increased between 11 and 20 wk gestation up to birth. Since hepatic Mn-superoxide dismutase activity did not change over this period, the increase was attributed to an elevated expression of CuZn-SOD. Liver GPx activities remained relatively constant during the same period, while hepatic CAT activity, although constant during gestation, increased significantly following birth. It appears that the expression of the antioxidant enzymes, CAT, SOD and GPx in lung and liver are not co-ordinated during human fetal development. These results do not support the concept of a concerted induction of lung antioxidant enzymes in utero in anticipation of the relative hyperoxia of birth. The varied patterns of expression throughout gestation, with CAT

activity increasing in the lungs and CuZn-SOD activity increasing in fetal liver, suggest that antioxidant enzyme activity in fetal tissue may vary in response to the metabolic needs of the tissue. These results also suggest that the developmental expression of antioxidant enzymes differs between tissues and that, unlike commonly used laboratory species, only the increased expression of catalase activity is associated with human lung development.

Gonzalez et al (101) reported physiological changes in the activities of the hepatic antioxidant enzymes CuZn and Mn-SOD and CAT, in the glutathione content and in the lipid peroxidation levels in fetal (day 20 and 21 of gestation) and neonatal rat liver (Days 1, 8, 15, and 22 post partum). They found CAT and SOD activities decreased before birth and increased after birth. The oxidized:reduced glutathione (GSSG:GSH) ratio declined before birth, but it increased between days 1 and 15 post partum and then remained stable. Finally, newborn rat liver from the first day of life showed the highest susceptibility to lipid peroxidation. These results suggest that the changes in antioxidant defences could be related to the beginning of food intake after birth, which entails a higher hepatic metabolism rate, as well as a higher oxygen consumption.

There are many factors that can affect antioxidant enzyme maturation. Van Golde et al (102) investigated the induction of AOE activity by hyperoxia (60% O<sub>2</sub>) in the developing chick embryo. They found that exposure to hyperoxia at different time points during incubation resulted in a 2-to 10-fold increase in SOD activity in all organs except the brain while CAT and GPx activities were only induced at 48 h after initiation of hyperoxia. Studies show that the response to hyperoxia depended on the kinds of AOE, organ, incubation time, and time points of exposure.

Bhandari et al (103) investigated the effects of hyperoxia on antioxidant enzyme activity in adult and fetal rat type II pneumocytes (TIIP). They found that in an *in vivo* study of hyperoxic exposure for 72 h in adult and neonatal TIIP, the adult TIIP had a significant



decrease in SOD but no changes in catalase or GPx activities. On the other hand, neonatal TIIP cells responded with an increase in SOD (170%) but no changes in GPx or CAT activities [104]. In an *in vitro* study of neonatal TIIP exposed to hyperoxia for 2 days [105], all AOE measured (SOD, CAT, and GPx) showed a decrease. The authors suggested that fetal TIIP would be resistant to oxygen toxicity by virtue of increasing AOE activity on exposure to hyperoxia.

The surfactant system and the antioxidant enzyme system undergo similar patterns of development, both exhibiting rapid increases in activity in the last 10 to 15% of gestation. These increases are essential for a smooth transition into neonatal life. While the surfactant system is necessary for alveolar stability, the antioxidant enzyme system protects against free radical injury. The development of the surfactant and AOE systems are regulated at least in part by glucocorticoid. The prenatal administration of glucocorticoids to enhance fetal lung maturation in threatening preterm deliveries is a well-established therapy. Sosenko et al (106) reported that glucocorticoid hormones can accelerate the maturation of the surfactant system and antioxidant enzyme system of the lung while thyroid hormone depresses antioxidant enzyme maturation in fetal rat lung, but increased lung tissue disaturated phosphatidylcholine (DSPC) and total phospholipid content. Ramadurai et al (107) also investigated the differential effects *in vivo* of thyroid hormone on the expression of surfactant phospholipid, surfactant protein mRNA and antioxidant enzyme mRNA in fetal rat lung. They found that T3 significantly increased DSPC content, and significantly decreased the expression of CuZnSOD, and CAT genes.

Carrillo et al (108) investigated age-related changes in antioxidant enzyme activities in rat and determined enzyme activities of SOD, CAT and GPx in the liver as well as several specific brain regions of young and old Fischer-344 rats of both sexes. They found that activities of CuZn-SOD were generally unchanged with age and activities of CAT as well as

GPx (both Se-dependent and non-Se-dependent forms) were also relatively unaffected by age.

In summary, the significant changes in antioxidant enzyme activity during gestation and development needs to be carefully interpreted, taking into consideration the fact that these changes are variable depending on species, the organs and the metabolic status examined.

### **1.3 The effects of hyperoxic exposure to antioxidant enzymes.**

Exposure of cells to hyperoxia leads to damage to the cells' molecular components and result in cellular dysfunction and death. In organisms that normally breathe air, the lung is the site of most damage during exposure to hyperoxia. When otherwise unmanipulated adult rats are exposed to > 95% O<sub>2</sub>, They begin to die at ~60 h of exposure and most are dead by 72 h (109, 110). Current evidence indicates that the damaging effects of hyperoxia are mediated by superoxide, hydrogen peroxide, and possibly the hydroxyl radical, moieties whose cellular production increases during hyperoxia (111, 112).

AOE protect against the damaging effects of oxygen. An increase in AOE activities during exposure to hyperoxia or an elevation produced before exposure and maintained during exposure, are associated with less O<sub>2</sub>-induced damage and fewer fatalities than occur without elevated antioxidant enzyme activity (113, 114). More direct evidence for a protective effect of AOE comes from genetic manipulations that cause the presence or absence of an AOE and result, respectively, in increased or decreased tolerance to O<sub>2</sub> (115, 116).

The ability to resist the potentially damaging effects of oxygen ( termed tolerance) varies among cells (117, 118) and between species (113). Tolerance can be age-dependent (119) and enhanced experimentally (120) and pharmacologically (121). In most

cases, age-dependent tolerance and tolerance induced pharmacologically or by experimental manipulation are associated with an elevation of lung antioxidant enzyme activity during exposure. In the following review, individual roles of AOE are discussed when exposed to high  $O_2$ .

### 1.3.1 Mn-SOD

Several studies show that Mn-SOD plays a very important role in protecting cells from hyperoxic damage. Clerch et al, (122) reported that elevation of lung Mn-SOD activity was essential for lung tolerance to hyperoxia in adult rats. The most compelling evidence is the following: (a) high  $O_2$  reduces Mn-SOD activities and increases more fatalities in old rats. The extent of the fall in Mn-SOD correlates with rat fatalities (123). (b) endotoxin can increase Mn-SOD activities and does not appear to effect CuZn-SOD, CAT and GPx activities. Endotoxin treatment leads to an increase in Mn-SOD activity and nearly 100% survival of adult rats during 72 h of exposure to 95%  $O_2$  compared to ~39% survival in untreated rats. These experiments show that Mn-SOD is involved in protecting animals from hyperoxia (124).

Other results have not supported this conclusion. White et al constructed transgenic mice that constitutively overexpressed CuZn-SOD and found that these mice were resistant to pulmonary damage by hyperoxia (125). In addition, Frank et al reported that treatment of rats with diethyldithiocarbamate (DDC), which inhibits CuZn-SOD but not Mn-SOD (122), substantially decreases endotoxin-induced tolerance.

Clerch et al (123) believed that these controversial results may be related to the differences in the ages of the animals used. Younger rats can easily increase their AOE activities and are tolerant to high  $O_2$  while older rats are vulnerable to high  $O_2$  because they can not increase their AOE activities. Therefore, varying responses to high  $O_2$  in

different animals could lead to different results. The mechanism for O<sub>2</sub>-induced decrease in Mn-SOD activities could also be due to a fall in its rate of synthesis (124) as Tsan et al (125) reported that the synthesis of Mn-SOD decreased in O<sub>2</sub>-exposed rats. In addition, increased degradation could contribute to the decline of Mn-SOD activity during exposure to hyperoxia (126).

### 1.3.2 CuZn-SOD

Several studies have reported that premature and newborn animals are more tolerant to oxidative stress than are adults (127, 128). It is possible that enzymatic defenses are capable of responding more rapidly and to a greater extent in younger animals than in adults when the organisms are exposed to oxidative stress (129). Hoffman et al (130) reported that CuZn-SOD activity in newborns increased after only 4 h of exposure to 95 % oxygen, but, in old rats, CuZn-SOD was not increased after 24 h of hyperoxia. Frank et al (131) reported that newborn guinea pigs and hamsters exhibited no increase in antioxidant enzymes under hyperoxic conditions and quickly died in this environment while newborn mice, rabbits and rats exhibited large increases in SOD, CAT and GPx and showed minimal lung damage after 7 days of exposure. These results suggest that the level of increase in antioxidant enzymes will determine survival after exposure to hyperoxia.

Allen et al (130) believed that much of the resistance of newborns to hyperoxia appears to stem solely from their ability to induce CuZn-SOD activity even though the roles of other antioxidant enzymes should not be ignored. When induction of CuZn-SOD is blocked with 0.25g/kg diethyldithiocarbamate (DDC), survival was decreased to under 40% in rats exposed to 95% oxygen. Autor et al (131) reported that the response of CuZn-SOD to high O<sub>2</sub> appears to be modulated by an unknown plasma factor. They found that neonatal rat lung tissue washed free of plasma and blood does not respond to hyperoxia. However,

if the tissue was treated with serum or plasma from normal human infants, CuZn-SOD and CAT activities did increase in response to hyperoxia. Treatment with plasma derived from human infants with hyaline membrane disease supported the increase in antioxidant enzymes. Although guinea pigs do not exhibit a rapid increase in CuZn-SOD activity in response to high  $O_2$ , treatment of rat or rabbit lung slices with guinea pigs sera permits a CuZn-SOD response to high  $O_2$ .

Qanungo et al (132) reported that the activity of CuZn-SOD in human placental homogenate and fetal liver increased significantly with gestational age. This increase in the activity of CuZn-SOD with the advancement of pregnancy indicated an increased protection of placenta and fetus against the damaging action of ROS and prepared for birth into an  $O_2$ -rich environment (133).

### 1.3.3 CAT

CAT plays a very important role in protecting tissues from hyperoxic damage. Experiments (134) have showed that high  $O_2$  exposure results in the generation of free radicals, including superoxide,  $H_2O_2$  and hydroxyl radical.  $H_2O_2$  must be scavenged by CAT or GPx in order to reduce high possible damage to cells. Without enough CAT to scavenge  $H_2O_2$ , the likelihood of cell survival is reduced. Spitz et al (134) believed that CAT is a major determining factor in cellular resistance to  $O_2$ . That group reported that  $H_2O_2$ -resistant cells were significantly resistant to acute  $O_2$  exposure (135, 136). When CAT was inhibited with 3-amino-1,2,4-triazole (AT), the cells were more sensitive to  $O_2$  exposure than were the control group. These results indicated that CAT activities might be a major determinant in the survival response to  $O_2$  exposure.

Nonetheless, CAT activity does not appear to be an important influence on  $O_2$  survival

responses during the initial stage of  $O_2$  exposure. Perhaps, at this stage, GPx plays a more important role in scavenging  $H_2O_2$ . Some researchers (134) believe that CAT activity may be important to the process of  $O_2$  toxicity once the overall cellular antioxidant defenses have been overwhelmed and cells begin the process of inactivation, but relatively unimportant in the initial period of acute  $O_2$  exposure. At this time, other antioxidant defenses (i.e. GPx and GSH) appear to be capable of compensating for the loss of CAT activity. Other researchers hypothesize that GPx may be relatively more important than CAT as an  $H_2O_2$  scavenger at lower concentrations of intracellular  $H_2O_2$  production based on a comparison of the differential compartmentalization of both CAT and GPx as well as kinetic considerations (137).

A higher resistance to hyperoxia of premature rats compared with adults has been repeatedly demonstrated and has been correlated with increased CAT activities (138). Sosenko et al reported that CAT activity increased by 34% in embryonic heart and lungs and 31% in the livers when exposed to hyperoxia. This increase in CAT activity helps to restore the balance between  $O_2$  radical production and antioxidant defenses. Premature guinea pigs were able to respond to a high  $O_2$  challenge with increased CAT activity while neonatal and adult guinea pigs demonstrated poor hyperoxic tolerance and failed to survive a prolonged  $O_2$  challenge because they were unable to mount an adaptive increase in AOE levels (139).

Newborn mammals are recognized as being more resistant to a variety of oxidative challenges than adult animals of the same species (140). The resistance of the neonatal tissues to oxidative damage depends on their capacity to exhibit an increase in antioxidant enzymes (139). Under normal conditions, induction of the antioxidant machinery present is expected to be enough to handle the increase of oxygen species formed as a consequence of the abrupt oxygen exposure immediately after birth, and to preserve the

structure and function of critical cell macromolecules against oxidative damage.

The activities of CAT in human placental homogenate, and fetal liver increases significantly with gestational progress (141). In order to meet the increasing demands of the growing fetus, more and more oxygen enters the feto-placental circulation during ontogeny thus exposing it to a greater threat of ROS. A steady rise in the activity of CAT throughout the gestational period is suggestive of a constant protection of these proliferating placental and fetal organs against the deleterious reactions of  $H_2O_2$ . The highest activities of CAT have been observed in fetal liver.

#### 1.3.4 GPx

Glutathione peroxidase is believed to play an important role in the cellular antioxidant defense by reducing hydrogen peroxide and various hydroperoxides using glutathione as a reducing agent to form water and corresponding alcohols.

Exposure of premature mammals to hyperoxia can cause extensive injury that result from the overproduction of ROS. However, it seems that most premature animals exposed to hyperoxia can adapt. Sosenko et al (142) reported that rats delivered 1 day prematurely had significantly increased activities of GPx and decreased indices of  $O_2$  toxicity. There are also contradictory reports. Ye-Shih et al (143) found that the mice deficient in cellular GPx-1 developed normally and showed no increased sensitivity to hyperoxia. These data suggest a very limited antioxidant role of GPx-1 in mice under normal physiological conditions and in defense against hyperoxia. The authors suggested that tissues could effectively decompose  $H_2O_2$  at both low and high concentrations of  $H_2O_2$ , presumable via catalase and other nonenzymatic mechanisms involving interaction between  $H_2O_2$  and cellular constituents such as lipids. Makino et al (144) reported that GPx played a major role in removing  $H_2O_2$  at relatively low  $H_2O_2$  concentrations in cells, and this function would

be taken over by catalase at high concentrations of  $H_2O_2$  to protect cells from injury.

A considerable body of evidence indicates that GPx increases in mammalian tissues during development as in the case of CAT. It is generally believed that this increase is caused by 1) the level of  $PO_2$ , 2) the rate of oxidative metabolism during fetal development, 3) by the maturational state of the tissues and by 4) the concentration of enzyme substrate (145). It is reasonable to expect that the premature animals could increase their GPx activity when exposed to hyperoxia.



## Chapter 2: Materials and methods

### 2.1 Introduction

At birth the fetus leaves a relatively hypoxic uterine environment to enter a relatively hyperoxic environment. This transition, involving a five-fold increase in oxygen concentration, is believed to pose a significant oxidative stress on the newborn through the increased production of oxygen free radicals. These partially reduced oxygen intermediates are capable of various cytotoxic effects.

Under normal circumstances tissue oxidative damage is kept to a minimum through the presence of an extensive array of antioxidant defenses. These include superoxide dismutase, catalase, glutathione peroxidase and other small molecular antioxidants. In the last few years, considerable evidence has accumulated to suggest that the fetus prepares for the oxidative stress of birth by rapidly synthesizing these antioxidant enzymes in the final stage of gestation. This has been considered a major part of the preparation for a successful transition to a higher oxygen environment at birth. In this study, we investigated changes in antioxidant enzyme activities in fetal rat livers during the final stage of development, from day 15 of gestation to day 22 of gestation, which in rats carried to term is the first day of birth. In addition, we also wished to determine: if inspired high  $O_2$  would increase AOE activities; and what roles SOD1 plays when full-term rat pups are exposed to high  $O_2$  concentrations. We hoped to get information from this rat model to provide baseline information and further our studies in human premature infants.

## **2.2 The purpose of the study**

- Investigate developmental changes of antioxidant enzyme activity (AOE) in fetal rat livers.
- Explore the relationships between CAT, GPx and SOD activities during development.
- Investigate the effects of high O<sub>2</sub> exposure on rat liver antioxidant enzyme activity.
- Investigate the role of SOD in high O<sub>2</sub> exposure.

## **2.3 Hypothesis**

- Antioxidant enzyme activities increase as gestation advances.
- The increase in enzyme activity change concomitantly for GPx, CAT and SOD.
- High O<sub>2</sub> exposure increases antioxidant enzyme activity.
- SOD plays an important role in response to high O<sub>2</sub> exposure.

## **2.4 Methods**

### **2.4.1 Experimental Design**

#### **2.4.1.1 Developmental changes in antioxidant enzyme activities.**

Timed-pregnancy Sprague-Dawley rats were ordered from Charles River Canada Inc. (Montreal, PQ). After one week of maintenance on standard laboratory food and water, these pregnant rats were either delivered on gestational day 15, 17, 19, 20 or allowed to deliver normally to term (day 22 of gestation). Two pregnant rats from each gestational day were anesthetized with sodium pentobarbital (Somnotol) intraperitoneally (1.5 mL/100 g bodyweight). A rapid hysterotomy was performed and fetuses were removed from the uterus. These fetuses were sacrificed with an overdose of somnotol

and their livers removed for AOE assay. The protocol was approved by the Animal Care Committee at Memorial University of Newfoundland.

**Table 1. Study design for developmental changes of AOE in rat liver**

Animals	Days of gestation at time of pup extraction				
	15	17	19	20	22
Pups	10	10	10	10	10

#### 2.4.1.2 The effects of O<sub>2</sub> exposure on AOE activities in full-term rat pups.

- Full-term rat pups were obtained from pregnant rats delivered on day 22 of gestation.
- Newborn rat pups were exposed to either high O<sub>2</sub> for 3, 6, and 9 days or to room air (controls).
- AOE activities were measured at days 3, 6 and 9 of exposure.

**Table 2. Study design for the effects of high O<sub>2</sub> exposure on hepatic AOE.**

Study Group	Room air	O <sub>2</sub>
Days exposed to high O <sub>2</sub>	3	10
	6	10
	9	10
Days exposed to room air	3	10
	6	10
	9	10

#### **2.4.1.3 The effects of DDC on hepatic AOE activities.**

- Pups were injected with DDC (150 mg/kg body weight, ip) at day 6 of exposure to room air or oxygen.

DDC inhibits CuZn-SOD by blocking the attachment of copper ions to the enzyme.

- AOE activities were measured at day 9 of exposure.

#### **2.4.2 Hyperoxic exposure**

Shortly after delivery, all full-term rat pups were pooled together and randomly redistributed to surrogate newly delivered (full-term) mother rats in chambers providing 95 % O<sub>2</sub> + 5% room air exposure. These chambers were continuously monitored for O<sub>2</sub> concentration. The temperature in the chambers was controlled between 23-26°C. The chambers were opened daily (10-15 min) for animal maintenance purposes, and the dams were switched from nursing high O<sub>2</sub> exposure litters to nursing room air litters in order to prevent the development of O<sub>2</sub> toxicity in the nursing dams. After 3, 6 and 9 days of exposure to either high O<sub>2</sub> or room air, pups were sacrificed with an overdose of somnotol intraperitoneally and their livers kept for AOE assays.

#### **2.4.3 Enzyme Analysis**

There are no certified reference enzyme assays currently available for the enzymes used in this study. Therefore initial assays were set up using established methods and comparing results to literature values. Results for all 3 enzymes fell within 15% of the values established for liver AOE available from the literature.

##### **2.4.3.1 Sample Preparation**

One hundred mg of tissue sample was homogenized in 1 ml of 10 mM sodium phosphate buffer ( pH 7.4, containing 3 mM KCl ), using an Ultra Turex homogenizer.

The resulting suspension was then sonicated for 45 seconds, in short bursts using a Soniprep 150. The crude tissue homogenate was centrifuged for 5 min at 13,500 g. The cytosolic supernatant was stored at -20 °C for subsequent antioxidant enzyme activity analysis. All enzyme determinations were carried out within 1 week of sample collection.

#### **2.4.3.1. SOD activity assay**

##### **2.4.3.1.2 Procedure**

The assay used was the pyrogallol autoxidation method described by Marklund and Marklund (146), which follows the rate of pyrogallol autoxidation at 420nm. For the assay of Mn-SOD, KCN was added to the sample in order to inhibit CuZn-SOD activity. The rate of pyrogallol autoxidation is highly dependent on the oxygen concentration of the solution, and equilibration of the buffer with air is therefore important. The rate of autoxidation is approximately linearly dependent on pyrogallol concentration. Using a standard curve prepared with purified CuZn-SOD from bovine erythrocytes (Sigma), one unit of SOD activity is defined as the amount of SOD that inhibits the autoxidation by 50% under these conditions.

Briefly, using a recording spectrophotometer with the thermostat set at 25 °C, the chart speed was set to 3 cm/min and the wavelength to 420 nm. Three ml of air-equilibrated buffer (50 mM Tris-HCl pH 8.2 containing 1mM DTPA, (diethylene triamine pentaacetic acid, to suppress interference by iron, copper and manganese ions) at 25 °C, with 10 µl catalase (30 µM CAT, Sigma) was added to the cuvette with either sample or blank. Contents were mixed, the recorder started, and 25 µl pyrogallol (24 mM Pyrogallol in 10 mM HCl) was added and mixed with a spatula. To measure cyanide-resistant SOD activity, we proceeded as above, except for adding 10 µl cyanide (0.3 M

KCN in distilled water) instead of 10  $\mu$ l catalase. Two different sets of samples and blanks were run, one set with catalase and one set with cyanide.

#### 2.4.3.1.3. Calculation

$$\text{Total SOD Activity (U)} = \% \text{INHIB}/50 \times 3 \times 1000/\text{Sv} \times 1/\text{PROT}$$

Where: one unit of SOD activity is defined as the amount of SOD that inhibits the autoxidation by 50% under these conditions; percent inhibition (%INHIB/50) is the rate of the reaction normalized to U; 1000= $\mu$ l / ml; 3 = volume in cuvette (ml); Sv = sample volume in  $\mu$ l; PROT=mg protein content per ml of sample. CuZn-SOD activity is calculated by subtracting the value for Mn-SOD from the total SOD value. The rate of the blanks is first estimated and the value was constant at about  $\Delta A_{420}=0.02/\text{min}$ . For maximum reproducibility, the best linear fit was drawn between  $\Delta A_{420}=0.02$  and 0.04 above baseline. The line that resulted was used for rate determination.

#### 2.4.3.1.4 General Comments

The rate of autoxidation is highly dependent on the oxygen concentration of the solution, and equilibration of the buffer with air is therefore important. Above pH 8, the rate of autoxidation increases 10 times for every increase in pH of 1 unit. Correct pH of the buffer is therefore important for reproducibility. The rate of autoxidation is almost always linearly dependent on the pyrogallol concentration.

#### 2.4.3.2 CAT

##### 2.4.3.2.1 Introduction

The spectrophotometric assay of catalase has been employed in the purification of catalase from both prokaryotic and eukaryotic sources because catalase catalyzes the decomposition of hydrogen peroxide as follows:



The decomposition of hydrogen peroxide catalyzed by catalase can be followed by an ultraviolet spectrometer, due to the absorbance of hydrogen peroxide at 240 nm. The diluted peroxide sample is recorded against a matched quartz cuvette containing deionized water

##### 2.4.3.2.2 Procedure

Briefly, catalase activity was measured according to the method of Beers and Sizer (1961). The rate of  $\text{H}_2\text{O}_2$  decomposition was followed by monitoring absorbance at 240 nm in 50 mM phosphate buffer, pH 7.0, containing 10 mM  $\text{H}_2\text{O}_2$  at 25°C. The specific activity of catalase is defined as:  $\mu\text{mol}$  of  $\text{H}_2\text{O}_2$  consumption per min per mg of protein. The absorbance of the diluted peroxide sample is then recorded against a matched quartz cuvette containing deionized water. The concentration of the stock  $\text{H}_2\text{O}_2$  is calculated from the molar extinction coefficient of  $43.6 \text{ M}^{-1} \text{ cm}^{-1}$ . The chart speed was set at 1 mm/sec and the reaction was monitored for 1 min. All measurement were repeated in triplicate.

##### 2.4.3.2.3 Calculation

Catalase specific activity (U) at 25 °C is defined in terms of  $\mu\text{moles}$  of  $\text{H}_2\text{O}_2$  consumed/min/mg of protein in the sample. The conversion of initial velocity (change in absorbance at 240 nm/min) to catalase specific activity is made as follows:

$$\text{Specific Activity} = \frac{\Delta A \times 1000}{43.6 \times \frac{\text{mg / protein}}{\text{mL}}}$$

Where 43.6 represents the molar extinction coefficient of  $\text{H}_2\text{O}_2$ .

### 2.4.3.3 GPx

#### 2.4.3.3.1 Introduction

Glutathione peroxidase catalyzes the GSH-dependent reduction of hydroperoxides:



This enzyme is an exceptional peroxidase due to its content of functional selenium, its substrate specificity (high for thiols but low for hydroperoxides), and its high reactivity with hydroperoxides. Its function as an integral part of the cellular protection mechanism against oxygen toxicity is well accepted and its activity has been used as a valid indicator of the selenium status of animals and humans.

In principle, each of the partial reactions, reduction of hydroperoxide or oxidation of GSH, could serve as a measure of glutathione peroxidase activity. The oxidation of GSH is the reaction normally monitored, while the more complicated approach required to measure hydroperoxide reduction is used for special cases, e.g., evaluation of kinetics. The enzymatic oxidation of GSH can be determined either by the time-dependent decrease in GSH concentration or the rate of GSSG accumulation. The former approach has proven better for the direct test procedure, while the latter provides the basis for the indirect, coupled test procedure, using the reaction of glutathione reductase as an indicator of GSSG production. In our research, we used the indirect procedure first described by Flohe and Gunzler (147). GSSG formed by the reaction of glutathione peroxidase is instantly and continuously reduced by an excess of glutathione reductase activity while providing a



constant level of GSH. The rate of NADPH oxidation relative to a given [GSH] is monitored spectrophotometrically at 365 nm.

#### 2.4.3.3.2 Procedure

Briefly, the enzyme reaction was conducted in a buffer containing 20 mM potassium phosphate, pH 7.0, 0.6 mM EDTA, 0.15 mM NADPH, 4 units of glutathione reductase (Sigma), 2 mM GSH, 1 mM sodium azide, and 0.1 mM H<sub>2</sub>O<sub>2</sub> at 25 C. The rate of decrease in absorption of NADPH at 365 nm was followed. GPx activity (U) is defined as nmol of NADPH consumption per min per mg of protein at 2 mM GSH. The non-enzymatic blank reaction rate was correspondingly assessed by replacing the enzyme sample by buffer. In our hands, the blank value was 0.009 or below.

#### 2.4.3.3.3 Calculation

Calculation of GPx enzyme activity (U):

$$Activity(U) = \frac{0.868 \times \Delta[NADPH] \times V_t}{[GSH]_0 \times t \times V_s}$$

Where: GPx activity (U) is defined as nmol of NADPH consumption per min per mg of protein at 2 mM GSH;  $\Delta[NADPH]$  is the rate of change of absorbance at this [GSH]; V<sub>t</sub>: volume in the incubation mixture in milliliters; V<sub>s</sub>: sample volume in the incubation mixture in milliliters; "t" is the time in minutes; 0.868 is the value determined empirically by the author after derivitization of the original equation..

#### 2.4.3.3.4 Comments

The reaction rate determined in the indirect coupled test procedure depends on steady-state levels of GSH so that GSH concentrations used in the assay have to be carefully controlled. Any factor influencing GSH regeneration, e.g., significantly

decreasing glutathione reductase activity, will affect the determination. The indirect test procedure appears to be the method of choice for determination of glutathione peroxidase activity in biological material.

#### **2.4.4 Protein Estimation with the Folin-Ciocalteu Reagent**

Protein contents of samples were determined according to the Lowry method using commercially available BSA (Sigma) as a standard. The Lowry procedure is one of the most venerable and widely-used protein assays. Under alkaline conditions, copper complexes with protein. When folin phenol reagent (phospho-molybdic-phosphotungstic reagent) is added, the Folin-phenol reagent binds to the protein. Bound reagent is slowly reduced and changes color from yellow to blue.

#### **2.4.5. Statistical analyses.**

Results are presented as mean  $\pm$  SD of measurement on liver from 10 samples at each developmental stage. Statistical analysis was performed by 1-way ANOVA tests. Significance was assigned to  $p < 0.05$ .

## Chapter 3: Results

### 3.1 Developmental changes in antioxidant enzyme activities

The prenatal developmental patterns determined for the antioxidant enzymes, total SOD, Cu/Zn-SOD, Mn-SOD, CAT, and GPx in rat liver are shown in Figs 1-10. Our results showed that CAT activity increased from 1.6 U to 10.4 U/liver (fig. 1) when its activity is expressed in mg protein or from 1.3 U to 165.3 U (fig.6) when its activity is expressed per total liver weight from day 15 of gestation to day 22 of gestation before birth. GPx activity also increased from 0.01 U to 0.11 U when its activity is expressed in mg protein (fig. 5) or from 0.12 U to 1.79 U/liver when enzyme activity is expressed per liver (fig.10) from day 17 of gestation to day 22 of gestation. When SOD activity is expressed in mg protein, total SOD activity decreased from 52 U to 32 U (fig. 2) from 17 day of gestation to day 22 of gestation. However, when its activity is expressed per liver, total SOD activity increased from 13 U to 59 U (fig. 7). The change in Cu/Zn-SOD activity was similar to total SOD activity, namely, Cu/Zn-SOD activity decreased from 17 U to 15 U when its enzyme activity is expressed in mg protein (fig.4) and Cu/Zn-SOD activity increased from 7 U to 25 U when its activity is expressed per liver (fig. 9) from day 17 of gestation to day 22 of gestation. As for Mn-SOD, there was no clear change in its activity from day 15 of gestation to day 22 of gestation, when its activity is expressed in mg protein (fig. 3 ). However, Mn-SOD activity increased from 8 U to 34 U during this period of time when enzyme activity is expressed per liver (fig. 8).

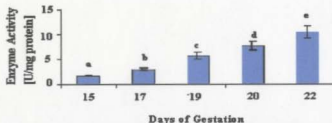


Fig. 1. The change of CAT activity in fetal rat liver during the last stage of the development. Groups with different letters are different from each other. Data are mean  $\pm$  SEM.

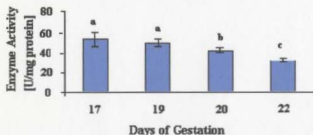


Fig. 2. The change of total SOD activity in fetal rat liver during the last stage of the development. Groups with different letters are different from each other. Data are mean  $\pm$  SEM.

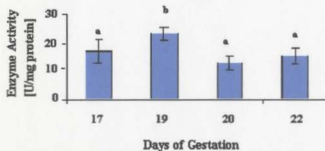


Fig. 3. The change of Mn-SOD activity in fetal rat liver during the last stage of the development. Groups with different letters are different from each other. Data are mean  $\pm$  SEM.

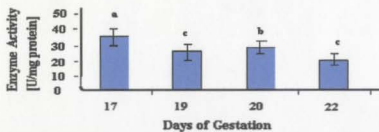


Fig. 4. The change of Cu/Zn- SOD activity in fetal rat liver during the last stage of the development. Groups with different letters are different from each other. Data are mean  $\pm$ SEM.

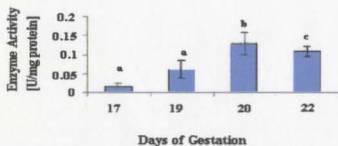


Fig. 5. The change of GPx activity in fetal rat liver during the last stage of the development. Groups with different letters are different from each other. Data are mean  $\pm$ SEM.

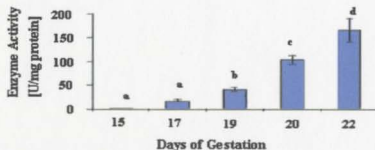


Fig. 6. The change of CAT activity in fetal rat liver during the last stage of the development. Groups with different letters are different from each other. Data are mean  $\pm$ SEM.

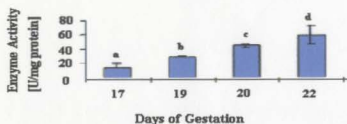


Fig. 7. The change of total SOD activity in fetal rat liver during the last stage of the development. Groups with different letters are different from each other. Data are mean  $\pm$  SEM.

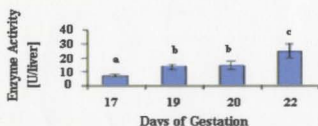


Fig. 8. The change of Mn-SOD activity in fetal rat liver during the last stage of the development. Groups with different letters are different from each other. Data are mean  $\pm$  SEM.

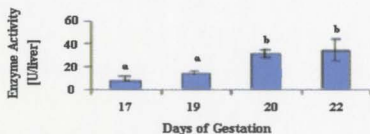


Fig. 9. The change of Cu/Zn-SOD activity in fetal rat liver during the last stage of the development. Groups with different letters are different from each other. Data are mean  $\pm$  SEM.

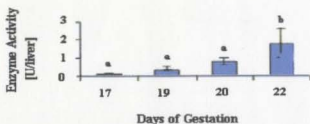


Fig. 10. The change of GPx activity in fetal rat liver during the last stage of the development. Groups with different letters are different from each other. Data are mean  $\pm$  SEM.

### 3.2 The effects of high $O_2$ on antioxidant enzyme activity in newborn rat liver.

All AOE (SOD, CAT, and GPx) activities increased in our experiments when newborn rats were exposed to 95%  $O_2$  for 9 days after birth. Both CAT and GPx activities increased continuously. CAT activity rose from 18 U/ mg protein to 85 U/mg protein (fig. 14) and GPx activity rose from 0.23 U/mg protein to 2.59 U/mg protein (fig. 15). However, the pattern of SOD activity was different from that of both CAT and GPX. Total SOD activity rose by 20% from 48 U/mg protein to 58 U/mg protein after 3 days of exposure and rose by 34% from 44 U/mg protein to 66 U/mg protein after 6 days of exposure, but only rose by 9% from 44 U/mg protein to 48 U/mg protein after 9 days of exposure compared with the corresponding newborn pups exposed to room air (fig. 11). The change in Cu/Zn-SOD activity increased significantly during the first 6 days of exposure similar to total SOD, and then decreased (fig. 13). There was no change in MnSOD activity during the first 3 days of exposure, but after 3 days of exposure, MnSOD activity began to increase significantly (fig. 12).

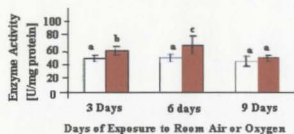


Fig. 11. The effects of high  $O_2$  on total SOD activity in newborn rat liver. The light bars represent room air, the dark bars oxygen exposure. Groups with different letters are different from each other. Data are mean  $\pm$  SEM.

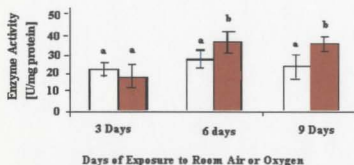


Fig. 12. The effects of high  $O_2$  on Mn SOD activity in newborn rat liver. The light bars represent room air, the dark bars oxygen exposure. Groups with different letters are different from each other. Data are mean  $\pm$  SEM.



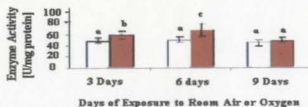


Fig. 13. The effects of high  $O_2$  on Cu/Zn-SOD activity in newborn rat liver. The light bars represent room air, the dark bars oxygen exposure. Groups with different letters are different from each other. Data are mean  $\pm$  SEM.

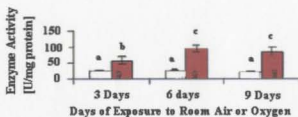


Fig. 14. The effects of high  $O_2$  on CAT activity in newborn rat liver. The light bars represent room air, the dark bars oxygen exposure. Groups with different letters are different from each other. Data are mean  $\pm$  SEM.

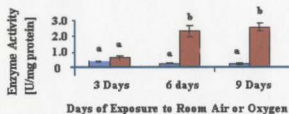


Fig. 15. The effects of high  $O_2$  on GPx activity in newborn rat liver. The light bars represent room air, the dark bars oxygen exposure. Groups with different letters are different from each other. Data are mean  $\pm$  SEM.

### 3.3 Inhibition of SOD by DDC

In our experiment, when the newborn rats were injected with DDC and exposed to high  $O_2$ , all rats died on the second day after injection. However, no rats died when they were only injected with DDC and exposed to room air or only exposed to high  $O_2$  without DDC injection. There were no changes in either CAT or GPx activities for rats who were only injected with DDC and exposed to room air (fig16 and 17). However, Cu/Zn-SOD activity decreased from 23U/mg protein to 11U/mg protein when the rats were only injected with DDC (fig.16). We did not see any change in Mn-SOD activity when the rats were only injected with DDC (fig. 16). Total SOD activity decreased as did Cu/Zn-SOD activity (fig. 16).

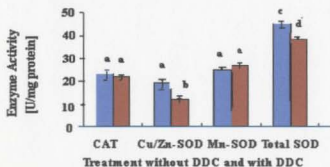


Fig. 16. The effects of DDC on CAT, Cu/Zn-SOD, Mn-SOD, and total SOD activity in newborn rat liver. The light bars represent the group without DDC, the dark bars with DDC. Groups with different letters are different from each other. Data are mean  $\pm$  SEM.

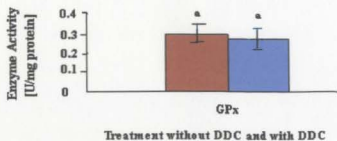


Fig. 17. The effects of DDC on GPx activity in newborn rat liver. The light bars represent the group without DDC, the dark bars with DDC. Groups with different letters are different from each other. Data are mean  $\pm$  SEM.

## Chapter 4: Discussion

### 4.1 The developmental change in AOE activity

At birth the fetus leaves a hypoxic environment to enter a relatively hyperoxic environment. This transition is believed to pose a significant oxidative stress on the newborn through the increased production of oxygen free radicals (161). However, under normal circumstances, tissue oxidative damage is kept to a minimum through the presence of an extensive array of antioxidant defenses. These include the enzymes SOD, CAT, and GPx. In order to be prepared for the relatively enriched O<sub>2</sub> world after birth, it is necessary that there is a prenatal increase and/ or a rapid postnatal response in AOE activity.

Frank et al first reported that SOD, CAT and GPx activities increased significantly in rabbits during the latter stage of development before birth and hypothesized that these increases in AOE were a "preparation for birth" (116). Other studies (106, 162, 163) have shown that AOE levels increase during the final part of normal gestation in several species including guinea pig, rat, hamster or human fetus. However, there are contradictory reports (109, 110, 125) that do not support the hypothesis by Frank. Gonzalez et al (125) reported that hepatic SOD and CAT activities decreased in rats before birth and subsequently increased after birth. How are these different reports explained?

Allen et al (164) suggested that some of the difference in AOE activity between species and between different investigators resulted from the differences in the methods as well as the units employed to quantify the activity of the enzymes. Activity has been variously expressed by different research groups in U/mg protein, U/g tissue, U/tissue and U/mg DNA. In most cases activity is expressed per mg protein. There is no consensus on which method is better used to describe the changes that occur in

enzyme activities. In the present study, we have noticed clear changes in the protein content of liver during development. When total protein levels increase more rapidly than enzyme activity, enzyme activities decrease on a U/mg protein basis. Therefore, we reported our enzyme activities both in U/mg protein and per whole liver. We think that in this way that we can clarify the changes in enzyme activities.

We chose the liver to study development of AOE because liver antioxidant capacity is 4-10 fold greater than lung and exhibits similar developmental patterns (161). In general development entails an increased metabolic rate promoting the generation of active oxygen species that could govern developmental changes. The activities of AOE could also be expected to increase in response to the upsurge in the rate of generation of cellular oxidants (113). To our knowledge this is the first report of the effects of hyperoxia on liver AOE activity.

In general, we found an increase in AOE before birth in rat liver homogenates from day 15 to day 22 of gestation. CAT activity increased from 1.6 U to 10.4 U /mg protein or from 1.3 U to 165.3 U/liver. GPx activity increased from 0.01 U to 0.11 U/mg protein or from 0.12 U to 1.79 U/liver. When SOD activity is expressed per/mg protein, total SOD activity decreased from 52 U to 32 U. However, when enzyme activity is expressed per whole liver, total SOD activity increased from 13 U to 59 U. In general, regardless of analytical method or units of expression, the general trend has been that SOD has been reported to increase in mammalian development (141), although the reason is unclear. Might there be a difference in interpretation of results depending on the way in which the units are expressed? Expressing enzyme activity per whole liver may be questioned as weight is made up of many factors including water and extraneous blood. And yet in our study, SOD activity expressed per whole liver supports the general trend of an increase in SOD with development. In general, therefore, we conclude that CAT, GPx and SOD activities expressed per whole liver increase significantly during the last stage of

development, whereas only SOD activity expressed per mg protein did not. What may these differences mean?

Some researchers (166, 167) have stated that the underlying cause of the increase in SOD during development was the induction of organisms into an oxygen rich environment which occurs at birth. Others (168, 169) have found that changes in SOD activity took place throughout the whole of development, not only limited to the final days before birth but continuing after birth. Allen et al (170) hypothesized that the development-associated changes observed in SOD activity were due to changes in the rate of cellular  $O_2^{\cdot-}$  production. They believed that the rate of free radical generation was low during the early stages of development and at the later stages, as more free radicals were produced, SOD activity would increase accordingly because the activity of SOD was sensitive to changes in the steady state level of  $O_2^{\cdot-}$ . Thus this process occurs before birth and may be separate from the effects of oxygen exposure after birth.

One other group reported findings similar to ours in developing rat liver. Utsumi et al (165) reported that SOD activity decreased from 74.3 U to 57.5 U/mg protein during the last stage of development from day 19 of gestation to day 22 of gestation yet increased in activity when expressed per whole liver (0.2 to 0.25 U). Indeed SOD levels were not adequate to protect liver exposed to aerobic conditions resulting in lipid peroxidation. We think therefore that reported increases in AOE activity are necessarily "preparation for birth" (104). It is reasonable that all AOE should increase in activity as they occur in the same metabolic pathways and that only changes in CAT and GPx activity are a preparation for birth without a similar concerted action of SOD. SOD catalytically removes  $O_2^{\cdot-}$  and produces  $H_2O_2$ , which is the substrate for the  $H_2O_2$ -removing enzyme, CAT and GPx.

Many studies (171, 172) have demonstrated that superoxide is an important signal molecule involved in signal transduction. From a signal transduction point of view, we

think that it is very appropriate to have a high concentration of signal molecules such as superoxide before birth to prepare and trigger developmental changes required for birth. A low level of SOD may be advantageous to the organism at this stage and be helpful in maintaining a high level of superoxide for signal transduction. This may explain the apparent inconsistencies in activity of AOE during the latter part of development prior to birth

Changes in the activity of catalase similar to our results, have been observed during the development of a number of different organisms. Frank et al (173) found that CAT activity began to increase at day 18 of gestation in rats. Mavelli et al (174) reported that CAT activity increased from 0.75 U/g liver at day 18 of gestation to 1.86 U/g liver at day 21 of gestation. Changes in mammals cannot be generalized for CAT as they are for SOD (141) and appear to be more tissue specific. For example Gonzalez (125) found a decrease in CAT activity before birth in rat liver in contrast to present results

A considerable body of evidence indicates that GPx also increases in mammalian tissues during development, and as in the case of catalase is tissue specific (141). Dillio et al (175) reported that in fetal rat liver, GPx activity increased from 2.22 U/g liver to 6.3 U/g liver from day 16 to day 20. Yoshioka et al (176) found that GPx in the rat lung tissue progressively increased from fetal to adult stages. Demis-Oole et al (177) demonstrated an increase in GPx activity of nearly 10-fold in adult rat liver homogenate as compared to neonatal liver homogenates. Higher GPx activity was observed in adult lung tissue from rats, rabbits, guinea pigs, mice, and hamsters as compared with neonatal tissue (178). These results and as well as those from the present study suggest that the increases in both CAT and GPx may be regarded as in protecting animals from damage by  $H_2O_2$  generated during the last stage of development before birth and possibly preparation for protection from oxygen exposure after birth.

Van Hien et al (168) demonstrated a progressive increase in SOD activity of human placenta during development. Nakagawara et al (169) reported that SOD activity tripled in human monocytes induced to differentiation in culture even though the oxygen concentration was kept the same during the experiment. It is likely that a coordinated increase in all 3 protective enzymes in lung may be required for maximum resistance to damage subsequent to hyperoxic exposure.

#### 4.2 The effects of high O<sub>2</sub> on AOE activity

It has been proposed that under ambient O<sub>2</sub> tension a biological balance exists between the normal production rate of O<sub>2</sub> radicals and the constitutive antioxidant defensive capacity of the cell (179). Exposure to elevated O<sub>2</sub> tension disrupts this normal biological balance by instigating excessive rates of O<sub>2</sub> radical generation including superoxide radical, hydrogen peroxide and hydroxyl radical. For our experiments we chose an exposure of 95% O<sub>2</sub> as that is the level of oxygen most frequently used to resuscitate the premature infant (179). As well, for pulmonary damage to be seen 60-70% oxygen exposure is required. Data from multiple animal model studies, both in vivo and in vitro, have strongly suggested that once oxidant-antioxidant imbalance is established, effective protection from oxidant-induced cell damage depends on the ability of the cell to respond to this biochemical threat by somehow augmenting its AOE activity levels. Animals or cells/tissues in culture which are capable of inducing this adaptive biochemical response to a hyperoxic challenge are usually found to be relatively tolerant to hyperoxia-induced cell damage and lethality (123, 124). Conversely, failure to manifest any increased AOE activity levels during hyperoxic exposure is usually associated with progressive damage and animal or cell/tissue lethality (125, 126). More direct evidence for a protective effect of AOE against overexposure to O<sub>2</sub>



comes from genetic manipulations that cause the presence or absence of an AOE and result, respectively, in increased or decreased tolerance to O<sub>2</sub> (127, 128). In general however, exposure of most mammals to hyperoxia results in progressive lung damage leading eventually to death (184). The effect of hyperoxia on liver AOE activity is less well known.

Frank et al (173) investigated the effects of high O<sub>2</sub> on AOE in lungs of full-term newborn rats, mice, rabbits, guinea pigs and hamsters. They found that high O<sub>2</sub> induced an increase in Cu/Zn-SOD activity by 33%, in MnSOD activity by 27%, in CAT activity by 34%, and in GPx activity by 85% in the rat lung after 6 days of exposure to O<sub>2</sub>. They believed that these increases in AOE activities played an important role in protecting newborn rats from pulmonary oxygen toxicity. In contrast, guinea pigs and hamsters did not show such increases in lung AOE activity and did not survive. Adult animals succumbed to O<sub>2</sub> toxicity in 3-5 days, suggesting this response in neonates was an adaptation to increased O<sub>2</sub> exposure at birth. Interestingly and of relevance to the premature infant, neonatal guinea pigs exposed to 95% O<sub>2</sub> were protected when given parenteral nutrition that enhance GSH production. Van Golde et al (181) also investigated the effects of high O<sub>2</sub> exposure on AOE activities in the developing chick embryo and found that hyperoxia induced AOE activities in all organs except the brain. They also noticed that the response to ROS was strikingly higher in the liver than in the other organs that confirms the extensive metabolizing and detoxifying capacity of liver. They suggested that further studies were required to determine whether this increase in AOE activities would be sufficient to circumvent cell damage.

We did not see changes in CAT and SOD in control animals exposed to room air during the first 9 days of life similar to the results of Keeney et al (128) who reported no rise in lung SOD after exposure to room air. This contrasted with the results of Gonzalez et al (125) who reported increases in AOE activity by day 8 which he attributed to higher

hepatic metabolism due to the effect of weaning. However, all AOE (SOD, CAT, and GPx) activities increased in our experiments when newborn rats were exposed to 95% O<sub>2</sub> for 9 days after birth. Both CAT and GPx activities increased continuously during this time period. CAT activity rose almost by 4 fold and GPx activity rose by ten fold during 9 days of exposure to high O<sub>2</sub>. However, the pattern of changes in SOD activity was different from that of both CAT and GPx. Total SOD activity rose by 20% after 3 days of exposure, rose by 34% after 6 days of exposure, but only rose by 9% after 9 days of exposure compared with the activity increase of newborns exposed solely to room air. Cu/Zn-SOD activity increased significantly during the first 6 days of exposure and then decreased. There was no change in MnSOD activity during the first 3 days of exposure, but after 3 days of exposure, MnSOD activity began to increase significantly. Several studies showed that Mn-SOD plays a very important role in protecting cells from hyperoxic damage. Clerch et al (146) reported that elevation of lung Mn-SOD activity was essential for lung tolerance to hyperoxia in adult rats. After 48 hours of hyperoxia, MNSOD activity initially decreased and then increased due to an increased synthesis rate measured by an increase in mRNA. The authors suggested that the increased synthesis rate was due to altered translational efficiency. Although we did not measure mRNA, these results in rat lung were similar to ours in liver and suggest an explanation for the delayed increase in MnSOD activity seen in our study.

Oxygen induced lung toxicity has been shown to be age dependent although the mechanism for this is unclear. Yam et al (182) reported that the neonatal animals can respond to hyperoxic exposure with significant increases in their pulmonary AOE levels and that the lungs of the adult animal failed to manifest increased AOE activity during similar hyperoxic exposure. In the lung young animals have a rapid pulmonary response compared to adults. Whether or not this would occur in liver has not been shown. In our experiment, SOD activity increased during the first 6 days when the animals were

exposed to hyperoxia, however there was no increase in SOD activity from days 6 to 9. We do not think this is an age response as the 3-day time difference was very short. Frank (143) suggests that neonatal rats lose their tolerance to hyperoxia at one month of age.

In the lungs of 10 day old rats maximal levels of SOD, CAT and GPX were reached within 24 hours of exposure to hyperoxia and were maintained until 4 days of age (184) as was seen in the present study. Hoffman (184) did not provide more than 4 days of hyperoxia so that we cannot tell from their data what would happen to AOE activity if rats were exposed to longer periods of hyperoxia. Keeney et al (128) reported that term rat pups exposed to hyperoxia lung CAT and GPx activities rose to maximum in 48 hours and stayed the same until 72 hours after which there was no further data. We think that the initial response in animals may be enough to protect them from damage and there is no need to respond further with increased activity or alternatively that the resources the young animal can draw on to resist hyperoxia are eventually depleted.

In lungs, the increase in AOE activity is the result of the *de novo* synthesis of protein (enzyme induction) and appears to occur after only 24 hours from initiation of hyperoxia. No such rapid induction of these enzymes has been found in adults of any species (184).

Both CAT and GPx play a very important role in protecting tissues from hyperoxic damage. Spitz et al reported (149) that high O<sub>2</sub> exposure resulted in the generation of free radicals, including superoxide, H<sub>2</sub>O<sub>2</sub> and hydroxyl radical. CAT or GPx must scavenge H<sub>2</sub>O<sub>2</sub>, in order to reduce high O<sub>2</sub> damage to cells. If there is not enough CAT or GPx to scavenge H<sub>2</sub>O<sub>2</sub>, it will be difficult for cells to survive. There was an increase in both CAT and GPx activity in our experiment when the newborn rats were exposed to high O<sub>2</sub>. We believe that the increases in both CAT and GPx activities play roles in protecting animals from attack from ROS and reflect an appropriate response. Indeed

baseline AOE levels are much less important in determining resistance to hyperoxia than are the responses of AOE to the hyperoxic challenge (179). Spitz et al (146) believed that CAT is a major determining factor for cellular resistance to  $O_2$  and reported found that  $H_2O_2$ -resistant cells are significantly resistant to acute  $O_2$  exposure (147, 148). When he inhibited CAT with 3-amino-1,2,4-triazole (AT), the cells were more sensitive to  $O_2$  exposure than the control group. These results indicated that CAT activities might be a major determinant of the  $O_2$  survival response after  $O_2$  exposure. However, our data suggests that both CAT and GPx responses were not as important in determining the survival of rat pups with high  $O_2$  exposure in our experiment as the increase in both CAT and GPx activities alone were not enough to ensure survival. For example, GPx null mice may still decompose hydrogen peroxide probably due to the presence of CAT, which may compensate (49). SOD appears to have a different response to hyperoxia depending on maturity of the animal (184). We believe that the increase in SOD activity is perhaps more important in helping animals to survive high  $O_2$  exposure as will be shown in the following section.

#### 4.3 Inhibition of SOD by DDC

Yam et al (182) investigated the effects of high  $O_2$  on the neonatal rats. They found the activities of all the AOE tested were significantly elevated in the neonatal rat lungs and no increases in the pulmonary non-AOE activities examined occurred during the  $O_2$  exposure period (including the enzymes alkaline phosphatase,  $\beta$ -glucuronidase, glutamic dehydrogenase, and fructose-1, 6-diphosphate aldolase). They carried out a follow-up study utilizing the SOD activity inhibitor, Diethyldithiocarbamate (DDC), and tested the importance of the AOE activity increases to the phenomenon of neonatal  $O_2$  tolerance. They found that when these neonatal rats received dosages of DDC sufficient to block any SOD increases during 95%  $O_2$  exposure, they evidenced loss of  $O_2$

tolerance and succumbed to O<sub>2</sub> toxicity. Diethyldithiocarbamate is an inhibitor of Cu/Zn-SOD as it is a copper chelator, and has been used to inhibit Cu/Zn-SOD and investigate the roles of Cu/Zn-SOD (183). Allen et al (142) believed that much of the resistance of newborns to hyperoxia appears to stem solely from their ability to induce Cu/Zn-SOD activity even though the roles of other antioxidant enzymes should not be ignored. When DDC blocked this induction, survival was decreased to less than 40% in rats exposed to 95% oxygen. In our experiment when newborn rats were injected with DDC and exposed to high O<sub>2</sub>, all rats died on the second day after injection. However, no rats died when they were only injected with DDC and exposed to room air or only exposed to high O<sub>2</sub> without DDC injection. These results demonstrate that Cu/Zn-SOD plays perhaps the major role in AOE activity for the enzymes that we examined, in the ability of animals to survive high O<sub>2</sub> exposure.

There are other cellular antioxidants that may play very important roles in development. For example, Allen et al reported that GSH concentration increases during the development of tissue with a high regenerative capacity (164). Maybe, the changes of GSH concentration is also involved in protecting fetuses from high O<sub>2</sub> damage after birth. As well, by not measuring lipid peroxidation as a measure of exposure to oxygen we have limited the interpretation of our results.

We believe that these increases in AOE activities played an important role allowing the experimental animals to survive high O<sub>2</sub> exposure because all the animals that were injected with DDC, died. This confirms that SOD plays a key role in protecting animals from high O<sub>2</sub> damage.

## Chapter 5: Conclusions

- Both CAT and GPx activities increased during the last stage of development when enzyme activity was expressed per protein.
- Total SOD activity decreased when enzyme activity was expressed per g protein.
- Total SOD activity did not change concomitantly with both CAT and GPx activity during the last stage of development.
- All liver antioxidant enzyme (SOD, CAT, GPx) activities increased significantly during exposure to high  $O_2$ , but total SOD activity declined after 6 days.
- Cu/Zn-SOD plays a key role for pups in surviving high  $O_2$  exposure. Once SOD is inhibited, no pups can survive.

## Chapter 6: References

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